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(54) Title: METHOD FOR DETECTING A VIRUS IN CELL MATERIAL OF A SCRAPE OR BODY FLUID

(57) Abstract

Method for determining a virus in cell material of a scrape or body fluid, by means of in situ hybridisation, characterised in that first a cell concentrate is prepared from a suspension of the scrape in a liquid medium, which suspension is first prepared in the case of a scrape, or from the body fluid, the cell concentrate is fixed and the fixed concentrate is embedded by means of the known histological embedding technique in paraffin or similar material, after which parts of the block obtained are subjected to the in situ hybridisation according to the known in situ hybridisation technique used with tissue sections.

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Method for detecting a virus in cell material of a scrape or body fluid.

The invention relates to a method for detecting a virus in cell material of a scrape or body fluid by means of in situ hybridisation.

The in situ hybridisation technique is an efficient technique for detecting the presence of viruses and is at present routinely utilised on biopsy material. The utilisation of this technique on scrapes on the other hand has little success in view of the problems which arise. With the known utilisation on scrapes these scrapes are directly smeared on a slide after which the in situ hybridisation is applied. There is however an absence of good adherence of the scrapes to the slide. Furthermore the amount of cytologic material is very limited while on the other hand a large amount of expensive test material is necessary in order to cover the slide, through which this method is very costly.

Nevertheless a better and cheaper method with utilisation of the in situ hybridisation technique could be very advantageous among others for detecting the papilloma virus (HPV) in cervical cells since this can be useful for making a diagnosis afterwards. The genome of this virus has after all been found in cervical neoplasms and a causal connection is suspected between the presence of this virus and cervical neoplasia. At present the presence of the HPV virus in scrapes from the cervix is detected with other techniques such as polymerase chain reaction. These techniques are,

like the known method with the in situ hybridisation of scrapes, not suitable for large scale examination of the female portion of the population for the presence of the papilloma virus.

The invention is intended to remedy the aforementioned disadvantages and to provide a method for detecting a virus in cell material of a scrape or body fluid whereby the in situ hybridisation can be utilised in an economic manner and without problems both with regard to the amount of cell material and to adherence to slides.

For this purpose first a cell concentrate is prepared from a suspension of the scrape in a liquid medium, which suspension is first prepared in the case of a scrape, or from the body fluid, the cell concentrate is fixed and the fixed concentrate is embedded by means of the known histological embedding technique in paraffin or similar material, after which parts of the block obtained are subjected to the in situ hybridisation according to the known in situ hybridisation technique used with tissue sections.

The applicant has in startling manner determined that, by not performing the in situ hybridisation directly on a smear of scrape but on cells which are suspended, concentrated, fixed and embedded in paraffin, the in situ hybridisation can be applied without problems in the same manner as with tissue sections whereby the detection of a virus in cells of a scrape, or of a body fluid such as blood, in which case the suspension is obviously superfluous, can take place in a relatively easy and inexpensive way with a minimum of scrape or body fluid.

In the case of a scrape, the suspension is preferably prepared with a phosphate buffered salt solution with a physiologic pH.

In a particular embodiment of the method according to the invention the cell concentrate is prepared by centrifuging, and the sediment obtained is further treated.

In a suitable embodiment of the method according to the invention, before forming the paraffin block, the cell concentrate is first embedded in a medium for forming a preparatory cell block and this cell block is later processed into a paraffin block.

This facilitates further operations and ensures a large number of cells in the paraffin block.

For the hybridisation slices of the paraffin block are preferably mounted on slides pre-treated with adhesive.

With the in situ hybridisation technique as utilised on tissue sections and which according to the invention is also utilised on paraffin block parts it is usual that the paraffin blocks are first de-paraffined, subsequently they are subjected to an enzymatic pre-treatment, denatured, and finally they are subjected to the actual in situ hybridisation, after which the parts are washed and the virus detected by means of test material.

Moreover use can be made of test kits available on the market destined for detecting a virus such as the papilloma virus in tissue.

Other details and advantages of the invention will appear from the following description of a method for detecting a virus in cell material of a scrape or body fluid according to the invention. This description is only given as an example and does restrict the invention.

For the determination of the papilloma virus in cell material which is present in a scrape from the cervix, this

scrape is put into suspension in a phosphate buffered salt solution with a physiologic pH. This suspension can temporarily be stored in a refrigerator.

Subsequently a cell concentrate is prepared by centrifuging the suspension at room temperature and the concentrate obtained, namely the sediment, is mixed with a viscous, hardly water-soluble medium such as an agar solution in distilled water at 60 degrees Celsius.

The solidified cell block which is obtained at room temperature after cooling off is put into neutral buffered formaldehyde solution (formalin) for fixing the cell material.

A paraffin block is prepared from this cell block with the assistance of a known technique which is utilised with tissue sections. Moreover the cell block is first dehydrated by means of alcohol which is thereafter removed by means of a solvent. Subsequently this solvent is substituted in the cell block by melted paraffin which is subsequently allowed to solidify by cooling off.

Thin sections, with a thickness of for example 5 micrometres, are cut from the paraffin block, and these sections are mounted on slides which are pre-treated with an adhesive such as 3-aminopropyltriethoxysilan. In between the slices can be allowed to float on a protein-free water bath. After drying the sections, they are maintained for 12 hours at a temperature of 58 to 60 degrees Celsius, whereby the paraffin melts and a good adherence to the slides is achieved, after which they are kept dust-free for the time desired.

The actual detection of the papilloma virus occurs according to the known in situ hybridisation technique, whereby the paraffin sections are treated in the same manner as tissue

sections which are already routinely treated according to this technique.

Moreover use is made of test kits which are on the market normally for detecting the papilloma virus in tissue sections.

A number of sections are de-paraffined beforehand by means of a solvent such as xylene, after which they are rinsed with alcohol and dried in the air.

Subsequently these sections are subjected to an enzymatic treatment with Proteinase K in order to liberate the DNA from the cells in the sections.

For each detection three test materials or probes are necessary of which a drop is applied respectively to three different slices. A first test material contains marked papilloma virus DNA sequences. A second, so-called positive DNA control test material, is specifically for human genome DNA sequences and generates a hybridisation signal in human cells. The third test material, the so-called negative DNA test material is specifically for a plasmid vector and normally produces no hybridisation signals. A vector sequence present in this last test material does not hybridise with the DNA in human cells.

The DNA to be tested must be denatured, which occurs by maintaining the section for five to ten minutes at 100 degrees Celsius. If the DNA in the test material contains one single strand this denaturisation can take place before the test material is added. If the DNA in the test material is double stranded, which is usually the case, then the denaturisation is performed after adding the test material.

The hybridisation is performed for 18 to 24 hours at 37 degrees Celsius in a humid incubation chamber. Thereafter

the excess test material is washed away from the sections.

For the actual detection of the virus, a drop of detection reagent is applied to each section. After incubation at 37 degrees Celsius a substrate solution is applied and, after washing, a solution for staining. After again washing and dehydrating in alcohol the section is mounted in a mounting medium.

The invention will be clarified hereafter on the basis of the following example:

The presence of condylomas was determined in five women by colposcopy and PAP smears showed the presence of koilocytes, which led to the assumption of an infection with the papilloma virus. The presence of this virus was confirmed via polymerase chain reaction.

The method according to the invention was subsequently applied to scrapes from the exo- and endocervix from these women, which scrapes were separately dissolved in ten millilitres of a phosphate buffered salt solution at a pH of 7.4.

Each suspension was put into a plastic tube of 50 millilitres and centrifuged at 1000 revolutions per minute for 10 minutes at room temperature. The sediment was mixed in a recipient with 0.1 millilitres of a 3% agar solution (bacteriological agar, GIBCO, U.K.) in distilled water at 60 degrees Celsius. After cooling the cell block was removed from the recipient and transferred to a plastic embedding cassette (Lancer, USA). Fixation was performed for two hours in neutral buffered formalin at room temperature.

The cell block was subsequently converted into a paraffin block by means of the usual histological paraffin embedding techniques with the assistance of a Tissue-Tek II

histokinette (Labtek, USA).

Sections of five micrometres were cut from the paraffin block which were allowed to float on a protein-free water bath. These sections were then adhered on slides pre-treated with 3-aminopropyltriethoxysilan (Digene, USA). After drying at 37 degrees Celsius the sections were heated to 60 degrees Celsius and maintained for a night at this temperature.

The enzymatic treatment with proteinase K and the hybridisation were performed according to the instructions from the manufacturer by means of the so-called "Viratype in situ HPV tissue hybridisation kit" in combination with the so-called "Viratype in situ HPV omniprobe set", both from Digene, U.S.A. The omniprobe set contained three DNA test materials or probes namely a DNA test material, marked with biotin, for the papilloma virus HPV 6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52 and 56, a positive control material specifically for frequently repeated sequences in the human genome and a negative control test material consisting of pBR 322 plasmid.

Beforehand each section was de-paraffinized in two changes of xylene, put into ethylalcohol at 95 degrees and dried in the air.

The proteinase K digestion solution from the aforementioned kit was applied to the sections and the sections were incubated for three minutes at 37 degrees Celsius.

After washing with a buffer, dehydrating in alcohol and drying in the air 3 slices were covered with 40 microlitres of respectively one of three aforementioned test materials and covered over with a covering plate. Subsequently the DNA of the test material and the DNA to be examined of the cell material of each slice to be examined were denatured at

100 degrees Celsius for 10 minutes by means of a "Präziterm" heating plate (Gestigheit, FRG).

The hybridisation was performed in a humid incubation chamber for 18 hours at 37 degrees Celsius. The covering plates were removed and the excess test material was rinsed away with a buffer.

Bonded test material was detected by means of a detection reagent bound with alkaline phosphatase. After washing with a buffer and incubating in a substrate solution of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium at 37 degrees Celsius for 60 minutes and again washing with distilled water the sections were treated with a staining solution (nuclear fast) for 30 seconds. After washing with distilled water, dehydration in 95 degree ethanol and rinsing in xylene the sections were mounted in a mounting medium Pertex (Histolab, Sweden).

Sections incubated with the HPV test material that detects HPV serotypes 6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52 and 56 showed a fine purple bluish granular signal in some nuclei of exocervical cells, mostly flattened cells presumably originating from the outer layers of the exocervical epithelium. With hematoxylin/eosin staining some of these cells had morphologic characteristics of koilocytes. The majority of the nuclei showed no reactivity and were faint pink red. Negative control sections incubated with pBR 322 plasmids showed no reactivity, which excluded the non-specific binding of the test material. Positive control sections showed in more than 90% of the nuclei a positive signal consisting of a purple bluish fine granular pattern.

The above described method is very reliable and fast. It only requires a small amount of test material, namely only 40 microlitres in order to cover a section with the cells,

as a result of which the method is also relatively inexpensive. Another great advantage consists in that from one paraffin block originating from one scrape a very large number of sections are obtained while only three sections are necessary for a test. This permits if necessary to repeat the test or to perform other processes on the remaining part of the paraffin block for determining the virus such as polymerase chain reaction in order to confirm the test. It is further possible if desired to sub-typify the papilloma virus or to isolate the DNA from the remaining material of the paraffin block.

Because of the fact that the method is relatively inexpensive and requires only little cell material it is possible to apply population screening.

The invention is in no way limited to the above described embodiment, and within the scope of the patent applications many changes can be made to the described embodiment.

In particular the method is not limited to the detection of the papilloma virus in cell material. Other viruses such as the Epstein-Barr virus or the cytomegalovirus can be detected according to the method.

The method is also not limited to the testing of scrapes. It can also be applied to body fluids such as blood or a broncho-alveolar rinse.

Claims.

1.- Method for determining a virus in cell material of a scrape or body fluid, by means of in situ hybridisation, characterised in that first a cell concentrate is prepared from a suspension of the scrape in a liquid medium, which suspension is first prepared in the case of a scrape, or from the body fluid, the cell concentrate is fixed and the fixed concentrate is embedded by means of the known histological embedding technique in paraffin or similar material, after which parts of the block obtained are subjected to the in situ hybridisation according to the known in situ hybridisation technique used with tissue sections.

2.- Method according to the preceding claim, characterised in that in the case of a scrape, the suspension is formed with a phosphate buffered salt solution with a physiologic pH.

3.- Method according to either one of the preceding claims, characterised in that the cell concentrate is prepared by centrifuging, and the sediment obtained is further treated.

4.- Method according to any one of the preceding claims, characterised in that before forming the paraffin block the cell concentrate is first embedded in a medium for forming a preparatory cell block and this cell block is later processed into a paraffin block.

5.- Method according to the preceding claim, characterised in that an agar solution in distilled water is used as medium.

6.- Method according to the preceding claim, characterised in that the cell block is dehydrated while processing it into a paraffin block.

7.- Method according to any one of the preceding claims, characterised in that for the hybridisation sections of the paraffin block are mounted on slides pre-treated with adhesive.

8.- Method according to any one of the preceding claims, characterised in that with the in situ hybridisation technique the paraffin blocks are first de-paraffined, subsequently they are subjected to an enzymatic pre-treatment, denatured, and finally they are subjected to the actual in situ hybridisation, after which the parts are washed and the virus detected by means of test material.

INTERNATIONAL SEARCH REPORT

International Application No CT/BE 92/00033

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; C12Q1/70

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JOURNAL OF CLINICAL MICROBIOLOGY vol. 27, no. 11, November 1989, WASHGINGTON,D.C.,USA pages 2429 - 2432 C.A.GLEAVES ET AL. See the whole document, especially p. 2429, left column, lines 24-32 and right column, lines 22-30 ---	1
X	WO,A,9 002 204 (RESEARCH DEVELOPMENT CORP.) 8 March 1990 see page 34, line 15 - line 25 ---	1
A	WO,A,9 010 715 (MOLECULAR BIOSYSTEMS) 20 September 1990 see the whole document ---	1

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 NOVEMBER 1992	14. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8 902 934 (MICROPROBE CORP.) 6 April 1989 see the whole document	1
A	DD,A,264 767 (AKADEMIE DER WISSENSCHAFTEN DER DDR) 8 February 1989 see the whole document	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. BE 9200033 SA 62820

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/11/92
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9002204	08-03-90	AU-A-	4652889	23-03-90
		EP-A-	0357437	07-03-90
		EP-A-	0440749	14-08-91
		JP-T-	4502553	14-05-92
WO-A-9010715	20-09-90	AU-A-	5289990	09-10-90
		CA-A-	2011571	07-09-90
		EP-A-	0462221	27-12-91
WO-A-8902934	06-04-89	AU-A-	2785589	18-04-89
		EP-A-	0338067	25-10-89
		JP-T-	2501442	24-05-90
DD-A-264767		None		

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